Severe pulmonary embolism decreases plasma L-arginine
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Take home message:
More severe PE reduced L-arginine and increased dimethylarginine concentrations, which could reduce NO biosynthesis.
Background: We hypothesize that pulmonary embolism (PE) with tricuspid regurgitation (TR+) causes intracardiac hemolysis, increasing plasma arginase-1 and decreased plasma L-arginine, the substrate of endothelial nitric oxide synthase (eNOS), together with increased synthesis of dimethylarginine metabolites which inhibit eNOS. Methods: Case-control study of normotensive patients with suspected PE who underwent CT pulmonary angiography and transthoracic Doppler-echocardiography. Significant tricuspid regurgitation (TR+), was defined by a doppler jet velocity > 2.7m/s. L-arginine was assayed by HPLC, arginase-1 by ELISA, and asymmetric dimethylarginine (aDMA) with mass spectrometry. Erythrocyte carbonic anhydrase-1 concentration was measured with ELISA as a biomarker of hemolysis. P<0.05 by ANOVA with Tukey’s post-hoc was considered significant. Results: Patients with PE+ and TR+ had significantly elevated mean plasma values of arginase-1, (24 ±8 ng/mL for PE- vs. 42 ±12 ng/mL for PE+ and TR+), lower L-arginine (121 ±8 vs. 65 ±6 uM) and elevated aDMA (0.15 ±0.04 uM vs. 0.27 ±0.08 uM). Plasma carbonic anhydrase-1 was also significantly elevated in PE+ and TR+. Conclusions: Patients with PE+ and TR+ have lower plasma L-arginine coincident with higher arginase-1 concentrations and increased aDMA. This combination may worsen pulmonary vasoconstriction associated with PE and could represent a new therapeutic target.
Introduction

Measurements from humans and animal models with PE have demonstrated increases in multiple vasoconstrictive molecules, including prostaglandins, platelet activating factor and leukotrienes.\(^1\) Nitric oxide (NO), produced tonically by vascular endothelial nitric oxide synthase (eNOS), plays a pivotal role in maintaining a normal pulmonary vascular resistance under these conditions.\(^2\) These facts underlie the construct hypothesis that in different subjects, PE can obstruct the same degree of pulmonary vasculature, but manifest widely different pulmonary arterial resistances.

Preliminary evidence from animal models and humans suggests that acute PE is associated with intravascular hemolysis, related to the severity of PE.\(^3\)\(^-\)\(^8\) Intravascular hemolysis liberates hemoglobin and diffusible heme which both directly bind NO. Ruptured erythrocytes also release large amounts of the enzyme arginase-1, which cleaves the eNOS substrate, L-arginine, producing urea and L-ornithine.

We hypothesized that patients with acute PE that causes significant tricuspid regurgitation will have acutely increased plasma concentrations of arginase-1, decreased L-arginine and increased aDMA compared with patients who have mild PE without tricuspid regurgitation as well as patients without PE.

Methods

Study Design: This was a secondary analysis of a four center prospective study of diagnostic accuracy conducted in patients with suspected PE (NCT00368836).\(^9\) The enrollment and diagnostic criteria have been described.\(^9\) Acute PE was considered present if two independent board certified radiologists interpreted a filling defect consistent with acute PE on computerized tomographic pulmonary angiography. Echocardiography was performed using techniques as previously
described, including pulse wave Doppler interrogation of the tricuspid regurgitant jet velocity measured >2.7 m/S, corresponding to an estimated right ventricular systolic pressure of 40 mm Hg.

**Blood collection**

L-arginine concentrations were assayed on thawed, citrate-anticoagulated plasma (0.11 mM) using high performance liquid chromatography. L-arginine standards (Sigma, St. Louis, MO) and unknown samples were precipitated with equal volume of 10% perchloric acid and the neutralized supernatants were mixed with Na₂BO₄, l-norleucine, NaCN, and Naphthalene 2,3 dicarboxaldehyde (dissolved in HPLC acetonitrile) and 100 uL aliquots were resolved in a 30-60% acetonitrile gradient mobile phase on a C-18 column using a Waters 616 LC System (Waters Corporation Milford, MA) L-arginine peaks were identified by location of external and internal arginine standards, and peak areas were corrected for quench of plasma and for stability of the derivitized l-arginine peak by the area of the internal standard, l-norleucine peak. Concentrations were determined from a standard curve for L-arginine using the Empower software package for L-arginine. Plasma arginase-1 and hemoglobin protein concentrations were measured using commercially available enzyme-linked immunoabsorbant assays (Human Arginase-1 I ELISA kit, Cat No: HK322. Hycult Biotech, Plymouth Meeting, PA; Human Hemoglobin ELISA kit, Cat No: E88-135, Manufacturer: Bethyl Laboratories, Montgomery, TX). Plasma carbonic anhydrase-1 (CA-1), was used as an additional biomarker of hemolysis, and was measured with a custom made sandwich ELISA. Briefly, biotinylated monoclonal mouse anti-CA-1 capture antibodies (Abcam, Inc. #34567) were immobilized to ELISA plate well bottoms (Nunc #439454), and incubated with 100 uL by plasma sample, washed, incubated with biotinylated goat polyclonal anti-CA-1 antibody and streptavidin conjugated to horseradish peroxidase as a chromogenic tag (450 nm). Standard curves were constructed using human CA-1 (#4396 Sigma Chemical, St. Louis, MO). Serum troponin I concentrations were measured with the iSTAT point of care system with an abnormal value considered >0.07 ng/mL (Abbott Point of Care Inc., Princeton, NJ).
Total dimethyl arginine content was assessed using tandem HPLC-MS with heavy arginine as a standard and assuming a 1:1 racemic balance for asymmetric and symmetric methyl arginine enantiomers. Solid phase extraction of plasma samples with heavy aDMA internal standard were performed using an Oasis MCX Cartridge (Waters Corp., Milford, MA). Components were separated by UPLC (NanoACQUITY UPLC, Waters, Milford, MA) and quantified using GC-MS/MS (TSQ Quantum XLS, Thermo Scientific, Waltham, MA).

**Statistical analysis**

Data were tested for normality (Shapiro-Wilk). P<0.1. Normally distributed data were compared with a one-way analysis of variance (ANOVA) with multiple comparisons using Tukey’s post-hoc test. Medians were compared using the Kruskal-Wallis test with pairwise comparisons using the Conover-Inman test. P<0.05 was considered significant [StatsDirect (Cheshire, England, v 2.6.2)].

**Results**

We enrolled 109 patients with PE including 44 with PE causing a mean pulmonary vascular obstruction of 33±28%. Doppler assessment found an abnormally high tricuspid regurgitant jet velocity (>2.7 m/S) in 20 patients (PE+TR+). Patients with PE were similar terms of age and comorbidities compared with patients who did not have PE. The troponin I concentration, which evidences increased PE severity, was elevated in 12/20 (60%) PE+TR+ patients, 2/24 (8%) PE+TR- patients, and in 2/65 (3%) PE- patients.

Patients with PE+TR+ had higher median free plasma carbonic anhydrase-1 [70.5 ug/L (IQR=37.2-13.6)] than PE- patients [50 ug/L (23-137)]. Patients with PE+TR- had elevated haptoglobin [2124 mg/L (1466-2621)], reflecting an acute phase increase in the setting of acute thrombosis, and haptoglobin was significantly lower with PE+TR+ [580 mg/L (468-678)] than PE+TR-, but was not
significantly lower than controls [888 mg/L (642-1362)]. The plasma free hemoglobin concentrations were not significantly different between groups, suggesting effective haptoglobin scavenging.

Figure 1 shows bar graph data of the mean (±SE) values of A. arginase-1, B. L-arginine, C. and the aDMA concentrations. Patients with PE+TR+ had higher arginase-1 and aDMA, and lower L-arginine values than patients without PE (P<0.05, ANOVA, Tukey’s post-hoc).

DISCUSSION
This study documents the decreased L-arginine blood concentrations in patients with PE that was severe enough to cause a TR jet velocity > 2.7 m/S. These patients also had increased free plasma concentrations of the erythrocyte enzymes carbonic anhydrase-1, arginase-1, and increased dimethyl arginine, together with decreased L-arginine compared with control patients who had no PE. Patients with PE and minimal or no TR had values of these biomarkers similar control patients without PE. Cases (PE+) and controls (PE-) were well matched in terms of age and comorbidities. These data provide evidence that acute PE with tricuspid regurgitation is associated with hemolysis, depression in the substrate for eNOS, and increase in an inhibitor of eNOS.

The hypothesized timing and location of shear upon erythrocytes with acute PE would require release of only a small amount of free hemoglobin to cause clinically important pulmonary vasoconstriction. The pulmonary vasoconstrictive effect of hemolysate has been well documented in isolated lung preparations. Upon its rupture, the erythrocyte releases tetrameric (α2β2) hemoglobin, which can immediately and avidly bind NO, but hemoglobin must first dissociate into αβ dimers before haptoglobin can bind and inactivate this NO scavenging effect—an effect that may require a few seconds to occur. In contrast to the millimolar concentrations required to constrict
peripheral vasculature, free hemoglobin in the low micromolar concentration will significantly increase pulmonary vascular resistance by nitric oxide scavenging.\textsuperscript{5, 12}

In conclusion, this report presents the first published data from humans to show decreased plasma L-arginine in patients with more severe PE, together with evidence of hemolysis. These data are consistent with the hypothesis that intravascular hemolysis contributes to pulmonary hypertension in patients with acute PE.
Reference List


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Figure 1 Plasma concentrations of A. Arginase-1 (**P<0.05 vs. PE+TR- and PE-, Tukey’s post-hoc); B. L-arginine (*P=0.03 vs. PE-) and C. Asymmetric dimethylarginine (*P=0.04 vs. PE-).
(Abbreviations PE pulmonary embolism, TR tricuspid regurgitation)